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Communication

Enzymic Synthesis of Caffeoylglucaric Acid from Chlorogenic Acid and Glucaric Acid by a Protein Preparation from Tomato Cotyledons[1]

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DIETER STRACK*, WILTRUD GROSS, VICTOR WRAY, AND LUTZ GROTJAHN
Botanisches Institut der Universität zu Köln, Gyrhofstrasse 15, D-5000 Köln 41 (D.S., W.G.), *and Gesellschaft für Biotechnologische Forschung (GBF), Mascheroder Weg 1, D-3300 Braunschweig* (V.W., L.G.), *Federal Republic of Germany*

ABSTRACT

The phenylpropane metabolism of tomato (*Lycopersicon esculentum* Mill) cotyledons was investigated. The HPLC analysis revealed two hydroxycinnamic-acid conjugates as major components, identified as chlorogenic acid (5-*O*-caffeoylquinic acid) and caffeoylglucaric acid (2-*O*- or 5-*O*-caffeoyl-glucaric acid). Quantitative analyses indicated a precursor-product relationship between the chlorogenic and caffeoylglucaric acids. Protein preparations from tomato cotyledons were found to catalyze the formation of caffeoylglucaric acid with chlorogenic acid as acyl donor and free glucaric acid as acceptor molecule. This enzyme activity, possibly to be classified as hydroxycinnamoylquinic acid:glucaric acid hydroxycinnamoyltransferase, acts together with hydroxycinnamoyl-CoA: quinic acid hydroxycinnamoyltransferase.

Tomato plants accumulate various caffeic acid (3,4-dihydroxycinnamic acid) depsides, positional isomers of caffeic acid esters of quinic acid (10, 18) and glucaric acid (3). To date two mechanisms for the formation of such esters have been described (1); one involving the hydroxycinnamoyl-CoA thioester and the other one the 1-*O*-(hydroxycinnamic acid)-acyl glucoside. The former mechanism was first shown to operate in the formation of chlorogenic acid (12), the 5-*O*-caffeoylquinic acid (6), in *Nicotiana* cell-suspension cultures and the latter one in the formation of the sinapic acid (4-hydroxy-3,5-dimethoxycinnamic acid) ester of malate (17) in *Raphanus* cotyledons. Both pathways can even lead to the same product, depending on the source of enzyme used, since recently it was shown that *Ipomoea* root tissue catalyzes the formation of chlorogenic acid from 1-*O*-caffeoylglucose (19, 20).

We studied the metabolism of hydroxycinnamic acid conjugates in tomato expecting one of the two described biosynthetic mechanisms to be operative in the formation of caffeoylglucaric acid. Earlier work (11) had shown that chlorogenic acid in tomato was formed via the caffeoyl-CoA thioester. However, all attempts to find an analogous reaction for the formation of caffeoylglucaric-acid, as well as experiments using 1-*O*-caffeoylglucose as possible acyl donor, failed. The latter mechanism was considered possible for the formation of caffeoylglucaric acid in *Cestrum* leaves which contain metabolically active 1-*O*-caffeoylglucose (9).

Since the accumulation patterns of chlorogenic and caffeoylglucaric acids indicated a precursor-product relationship, we considered chlorogenic acid as the possible acyl donor as was found in the biosynthesis of isochlorogenic acid (3,5-di-*O*-caffeoylquinic acid) in *Ipomoea* root tissue (8). Our results show that the enzymic formation of caffeoylglucaric acid in tomato cotyledons proceeds exclusively with chlorogenic acid as the acyl donor.

MATERIALS AND METHODS

Plant Material. Tomato seeds (*Lycopersicon esculentum* Mill cv Moneymaker Spezialzucht) were obtained from Waltz, Stuttgart, FRG; seedlings were grown for 3 weeks in a growth chamber with a 14-h day at 24°C and 70% RH in a defined soil as described (15). Adult plants were grown in a greenhouse.

Substrates. CoA (free acid), caffeic and chlorogenic acids (5-*O*-caffeoylquinic acid; IUPAC nomenclature [6]) were purchased from Fluka (Neu-Ulm, FRG), and quinic, glucaric, galactaric and gluconic acids from Merck (Darmstadt, FRG). Caffeoyl-CoA was synthesized chemically and identified according to Zenk and coworkers (4, 13) and purified by a modification (D Strack, H Keller, G Weissenböck, unpublished data) of a described method (7) involving the hydroxycinnamoyl-*N*-hydroxysuccinimide ester and subsequent transesterification of the hydroxycinnamoyl moiety onto CoA. Purification was achieved on a polyamide column (details not documented) by stepwise elution with water, methanol and increasing concentrations of aqueous NH_4OH in methanol (0.01%, 0.05%, 0.1%, 0.3%, 0.5%, v/v). The hydroxycinnamoyl-CoAs were recovered in the 0.3 to 0.5% ammoniacal-methanol fraction. Purity was checked by TLC on CAW (chloroform:acetic acid, 3:2, v/v, water saturated) and BAW (*n*-butanol:acetic acid:water, 6:1:2, v/v/v) and UV spectroscopy. 1-*O*-Caffeoylglucose was prepared by incubation of 10 g 28-h imbibed radish (*Raphanus sativus* L. var *sativus*) seeds for 15 h with 50 mM caffeic acid (dissolved in K-phosphate, pH 6.5). Isolation of the 1-*O*-caffeoylglucose—formed by the radish seedlings as detoxification product (5)—was accomplished by a described method (14). The product was identified by direct chromatographic comparison with 1-*O*-caffeoylglucose from petals of *Antirrhinum majus* (5).

Protein Preparation. Cotyledons (5 g) from 5 to 6 d old tomato seedlings were ground (15 min) in a precooled (0–4°C) mortar in the presence of 0.5 g Polyclar AT and 35 ml Tris-HCl buffer

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(100 mM, pH 8.0) containing 20 mM DTT and 1 mM EDTA. The homogenate was passed through Miracloth and the filtrate centrifuged for 30 min at 20,000*g*. The enzyme activity was precipitated by $(NH_4)_2SO_4$ (30–80% saturation) and redissolved in 2.5 ml K-phosphate (100 mM, pH 7.0) which was filtered through Sephadex G-25 (Pharmacia PD-10 columns). The eluate was used as source of enzymic activity. Protein content (5–6 mg/ml) was determined by the method of Bradford (2) using BSA as standard.

Enzyme Assay and Activity Determination. *Chlorogenic-acid formation.* The reaction mixture contained in a total volume of 120 μl K-phosphate (80 mM, pH 7.0) 8 mM DTT, 0.8 mM EDTA, 3 mM quinic acid (dissolved in 100 mM K-phosphate, pH 7.0), 0.1 mM caffeoyl-CoA (dissolved in water) and 25 μl protein solution. The control lacked quinic acid.

Caffeoylglucaric-acid formation. The standard reaction mixture contained 10 mM DTT, 1 mM EDTA, 4 mM glucaric acid, 30 mM chlorogenic acid (both substrates dissolved in 100 mM K-phosphate, pH 7.0) and 10 μl protein solution in a total volume of 100 μl K-phosphate (100 mM, pH 7.0). Control lacked glucaric acid. Apparent K_m and V_{max} values at fixed concentrations of the second substrate were obtained by Lineweaver-Burk plots.

Reactions were started by the introduction of the acceptor molecules (quinic or glucaric acid) and after incubation at 30°C for various periods of time were stopped by transferring the mixtures to a freezer (−20°C) or by immediate HPLC analyses. The liquid chromatograph (LKB) and the data processor (Shimadzu) have been described (16). Chromatographic conditions: linear gradient elution for 5 min from 30 to 50% solvent B (1.5% phosphoric acid, 20% acetic acid, 25% acetonitrile in water) in solvent A (1.5% phosphoric acid in water), then isocratic at 50% solvent B with a flow rate of 1 ml per min; detection at 320 nm; column (250 mm long, 4 mm inner diameter) prepacked with Nucleosil C18, 5 μm (Macherey-Nagel, Düren, FRG). The following R_t values were obtained: caffeoylgalactaric acid, 3.6 min; caffeoylglucaric acid, 4.4 min; chlorogenic acid, 8.2 min. Enzymically formed products were identified by co-chromatography (HPLC, TLC) with authentic chlorogenic and caffeoylglucaric acids.

Identification of Chlorogenic and Caffeoylglucaric Acids. *Chlorogenic acid.* Chlorogenic acid was identified in the crude extract by direct chromatographic comparison with reference material on HPLC (see above) and on TLC (microcrystalline cellulose, 'Avicel') with CAW (R_F 0.33) and BAW (R_F 0.45) as solvents. Caffeoylglucaric acid was isolated as follows. The 80% aqueous methanol (v/v) extract (1.5 L) from cotyledons (190 g) was vaccum concentrated to 4 to 5 ml and transferred onto the water-equilibrated polyamide column (CC-6 Perlon, 35 cm long and 4 cm inner diameter; Macherey-Nagel, Düren, FRG) which was extensively washed with water and methanol. Subsequent elution with 0.1% NH_4OH in methanol (v/v) gave chlorogenic and caffeoylglucaric acids. This fraction was chromatographed on microcrystalline cellulose in CAW and caffeoylglucaric acid (R_F 0.08; BAW, (R_F 0.16), which has a bright-blue fluorescence changing to a pale greenish blue when treated with ammonia vapor, was scraped off, eluted with methanol and rechromatographed on the polyamide column and finally purified on Sephadex LH-20 column (75 cm long, 3.5 cm inner diameter; Pharmacia, Freiburg, FRG) using methanol as solvent. The UV spectrum showed γ_{max}methanol at 327 nm with shoulder at 299 nm and γ_{max}methanol + NaOH at 373 nm with shoulder at 309 nm. Identification of the compound as 2-*O*- or 5-*O*-caffeoylglucaric acid was accomplished by NMR and MS spectroscopy. One- and two-dimensional ^1H NMR spectra were recorded at ambient temperature at 400 MHz on a Bruker WM-400 NMR spectrometer looked to the major deuterium resonance of the solvent, CD_3OD. Negative ion FAB[2] mass spectra were recorded on a Kratos MS-50 mass spectrometer equipped with a Kratos FAB source. Glycerol was used as matrix.

2-O- or 5-O-Caffeoylglucaric Acid. (See structure of the 2-*O*-isomer in Fig. 3). ^1H NMR (CD_3OD): δ = 7.675 [d; H-7′; J(7′-8′) 15.9], 7.113 [d; H-2′; J(2′-6′) 2.0], 7.002 (dd; H-6′; J(6′-5′) 8.2], 6.816 [d; H-5′)], 6.430 [d; H-8′], 5.254 [d; H-2 or 5; J(2-3) or (5-4) 5.5], 4.312 [dd; H-3 or 4; J(3-4) 3.0], 4.077 [d; H-5 or 2; J(5-4) or (2-3) 6.5], 3.922 [dd; H-4 or 3]. Negative ion FAB MS m/z: 372 [M-H]-, 209 [M-$C_9H_6O_3$]-.

Metabolite Quantification. Cotyledons from 10 to 20 seedlings at different developmental stages were homogenized with an Ultra-Turrax homogenizer in 2 ml 80% aqueous methanol for 2 min. The homogenate was allowed to stand for 30 min with stirring and then centrifuged for 10 min at 3,000*g*. The clear supernatant was subjected to HPLC (see above for product determination from enzyme assays) to separate and quantify the caffeic acid esters. Chlorogenic acid was used as external standard.

RESULTS AND DISCUSSION

Structure Elucidation of Caffeoylglucaric Acid and Accumulation of Caffeic Acid Esters. The HPLC analysis of 80% aqueous methanolic extracts of cotyledons from tomato seedlings showed two major secondary phenolic compounds, chlorogenic acid (5-*O*-caffeoylquinic acid) and caffeoylglucaric acid (Fig. 1). A third, minor compound was tentatively identified as caffeoylgalactaric acid by direct chromatographic comparison with the enzymically formed product from chlorogenic acid and galactaric acid (see below). The major compounds have been reported to occur in tomato leaves (3, 10). Co-chromatography of extracts from tomato cotyledons and leaves showed identity of the two depsides. Chlorogenic acid was readily identified in the crude extract by direct comparison with reference material. Caffeoylglucaric acid was isolated and its structure elucidated by NMR and MS spectroscopic analyses. A GLC comparison of the products from alkaline hydrolysis (data not shown; [3]) with standard com-

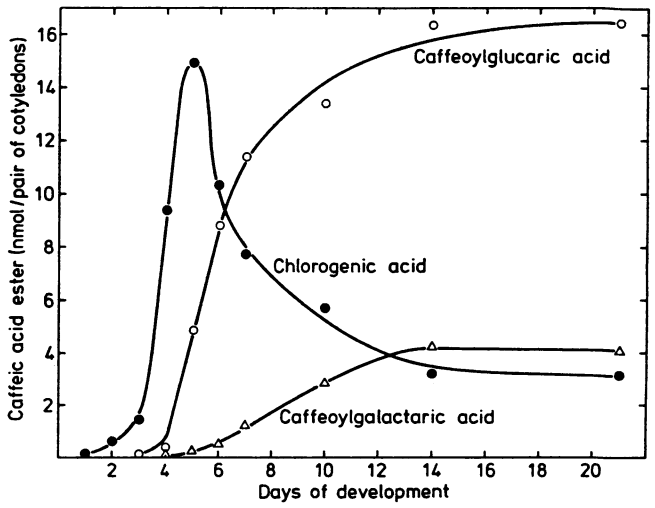

FIG. 1. Accumulation pattern of caffeic-acid esters in tomato cotyledons during germination (until d 3) and seedling development: chlorogenic acid (●), caffeoylglucaric acid (○), and caffeoylgalactaric acid (△). Caffeoylgalactaric acid has been tentatively identified by chromatographic comparison with the enzymically formed product from chlorogenic and galactaric acids. The data are the mean of two independent experiments.

[2] Abbreviation: FAB, fast atom bombardment.

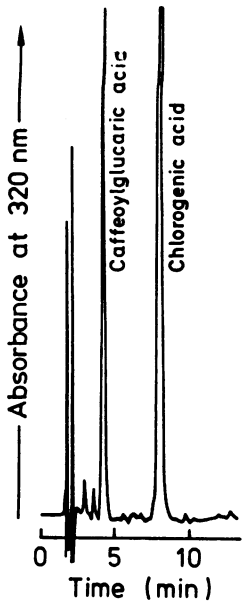

FIG. 2. HPLC analysis (0.032 absorbance unit full scale) of a transferase assay (2 h at 30°C) including 0.3% protein from tomato cotyledons, 0.2 mM chlorogenic acid as donor, and 3 mM glucaric acid as acceptor molecule. The product caffeoylglucaric acid (about 8% conversion) is being formed via the activity of a hydroxycinnamoylquinic acid:glucaric-acid hydroxycinnamoyltransferase.

pounds (glucaric and galactaric acids) showed identity with glucaric acid. In contrast to the simple negative ion FAB mass spectrum, showing a deprotonated ion at m/z 371 and a sequence ion FAB mass spectrum, showing a deprotonated ion at m/z 209 corresponding to the loss of the caffeoyl moiety, the complex one-dimensional ^{1}H NMR spectrum showed that the caffeoylglucaric acid was a mixture of isomeric structures in keeping with previous results on the same compounds (3, 9). In all, there were eight isomers present consisting of a *cis* (*Z*) and *trans* (*E*) mixture (1:2) of the four possible positional isomers. A two-dimensional ^{1}H COSY spectrum allowed ready identification of the spin systems of the various moieties. The major component, for which NMR data are given, was the *E* isomer (39%) of 2-*O*- or 5-*O*-caffeoylglucaric acid, readily identified from the multiplicity and low field shift of H-2 or H-5, and the characteristic vicinal coupling J(7′-8′). The question of the naturally occurring positional isomers of this depside was left unanswered in the previous studies on *O*-caffeoylglucaric acids (3, 9). The pseudo symmetry of glucaric acid does not allow distinction between position 2 and 5 (nor between 3 and 4) by NMR spectroscopy. The other seven components were present in proportions from 3 to 13%. There was no evidence from either NMR or mass spectroscopy for the presence of significant amounts of lactone forms. Co-chromatographic analysis (HPLC) of freshly prepared extracts with the isolated caffeoylglucaric acid showed that either the 2-*O*- or 5-*O*-caffeoylglucaric acid was the naturally occurring major compound. Appreciable amounts of the other positional isomers detected by NMR spectroscopy appeared during the isolation procedure (3).

The accumulation patterns of the caffeic-acid esters indicate a precursor-product relationship between chlorogenic acid and the caffeoylglucaric acid (Fig. 1). Chlorogenic acid accumulated to a maximum of 15 nmol per pair of cotyledons at d 5 of tomato-seedling development and decreased thereafter to 3 nmol until d 14. As the chlorogenic-acid level decreased the caffeoylglucaric acid accumulated to 16 nmol. The minor constituent, caffeoylgalactaric acid, accumulated to about 4 nmol and is also probably an *in vivo* product of the chlorogenic acid-dependent transferase

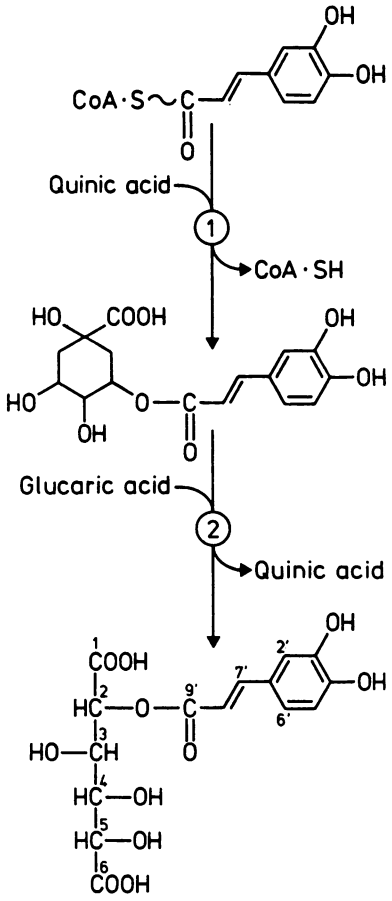

FIG. 3. Scheme for the enzymically catalyzed conversion of caffeoyl-CoA to caffeoylglucaric acid via chlorogenic acid as intermediate in tomato cotyledons. The structure of one of the possible two isomers formed, the 2-*O*-caffeoylglucaric acid, is shown. The involved enzymes are tentatively named hydroxycinnamoyl-CoA:quinic-acid hydroxycinnamoyltransferase ① and hydroxycinnamoylquinic acid:glucaric-acid hydroxycinnamoyltransferase ②.

activity (see below).

Enzymic Synthesis of Caffeoylglucaric Acid. Protein preparations from cotyledons of tomato (5–6 d old) were assayed for the enzymic syntheses of chlorogenic and caffeoylglucaric acids. As expected (11) the former compound was formed from the caffeoyl-CoA thioester and free quinic acid with an activity of about 18 pkat/per mg protein. However, experiments failed to find an analogous synthesis or caffeoylglucaric acid. Also a second possible mechanism via glucose carboxylic-group activation (1-*O*-acyl glucoside) (1), discussed to occur possibly in the caffeoylglucaric-acid formation in *Cestrum* leaves (9), could not be detected. A third possible mechanism involving the quinic-acid conjugate was therefore indicated. It was found that caffeoylglucaric acid was exclusively synthesized from chlorogenic acid and free glucaric acid, catalyzed by a chlorogenic acid-dependent acyltransferase (Fig. 2). This seems to be a stereospecific reaction, since there was only one product formed, showing chromatographic identity (TLC, HPLC) with the naturally occurring 2-*O*- or 5-*O*-caffeoylglucaric acid. Product formation was positively correlated with the amount of protein, and was linear for at least 60 min. Heat-denatured (5 min at 80°C) protein showed no enzymic activity. Recent studies on the acceptor specificity of the enzyme have shown that galactaric acid is also efficiently acylated by chlorogenic acid and the monocarboxylic acid gluconic acid is not accepted as a possible substrate. Saturation curves showed a normal Michaelis-Menten behavior for chlorogenic and glucaric

acids with respective K_m values of about 29 mM and 0.8 m*M*. The theoretical V_{max} value was calculated to be 146 pkat/mg protein.

Thus, our results indicate that in tomato cotyledons the caffeoylglucaric acid is being formed by the concerted action of two transferases (Fig. 3)—whose substrate specificities are not yet known—(a) hydroxycinnamoyl-CoA:quinic acid and (b) hydroxycinnamoylquinic acid:glucaric acid hydroxycinnamoyltransferases, preceded by hydroxycinnamic acid CoA-ligase ativity (11).

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